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Biofilm-Forming Ability of *Candida albicans* Is Unlikely To Contribute to High Levels of Oral Yeast Carriage in Cases of Human Immunodeficiency Virus Infection

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An increased prevalence of candidal carriage and oral candidiasis is common in cases of human immunodeficiency virus (HIV) infection, and the reasons for this may include the enhanced ability of colonizing yeasts to produce biofilms on mucosal surfaces. The aim of the present study was therefore to examine the differences, if any, in the biofilm-forming abilities of 26 *Candida albicans* yeast isolates from HIV-infected individuals and 20 isolates from HIV-free individuals, as this attribute of yeast isolates from patients with HIV disease has not been examined before. Biofilm formation in microtiter plate wells was quantitatively determined by both the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction method and the crystal violet method. Although candidal biofilm formation could be quantitatively evaluated by either technique, the better reproducibility ($P < 0.05$) of the XTT reduction assay compared with that of the crystal violet method led us to conclude that the former is more reliable. There were no significant quantitative differences in biofilm formation between *C. albicans* isolates from HIV-infected patients and isolates from HIV-free individuals during in vitro incubation in a multiwell culture system over a period of 66 h. Three of eight host factors in the HIV-infected group were found to be associated with candidal biofilm formation. Thus, yeasts isolated from older individuals and those with higher CD4-cell counts exhibited decreased biofilm formation, while the findings for yeasts from individuals receiving zidovudine showed the reverse ($P < 0.05$ for all comparison). Our data indicate that attributes other than biofilm formation may contribute to the increased oral yeast carriage rates in cases of HIV infection.

Candida biofilms have recently received considerable attention due to their high prevalence on catheter surfaces (1) and their notorious resistance to antifungals (14, 29). These characteristics of *Candida* biofilms are likely to contribute to both superficial (26) and systematic candidiasis (21). As candidal infections are the most common fungal infections in individuals infected with the human immunodeficiency virus (HIV) (27), we hypothesized that isolates recovered from HIV-infected individuals may have better biofilm-forming abilities than those recovered from HIV-free individuals.

A number of workers have compared the adherence of *Candida albicans* yeast isolates from HIV-infected patients and HIV-free subjects to mucosal surfaces, as adherence to host surfaces is a prerequisite for subsequent biofilm formation and colonization (12). However, those studies have yielded variable results showing enhanced adherence to buccal epithelial cells (BECs) by yeast isolates from HIV-positive individuals (32), a comparable degree of adhesion between test and control isolates (34), and an even higher degree of adhesion by control isolates (23). To our knowledge, no group has studied the biofilm-forming abilities of *Candida* isolates recovered from HIV-infected individuals. Furthermore, while factors such as CD4 count and the intake of medications, which are likely to affect candidal adhesion in individuals with HIV infection, have also been investigated in many studies (5, 13, 34), the association of these host factors with candidal biofilm formation in individuals with HIV disease has not been explored.

The availability of a rapid, inexpensive, and reproducible methodology for quantification of biofilm formation is essential for the evaluation of biofilm generation. A variety of direct and indirect quantitative methods have been developed for this purpose. Direct methods mainly consist of gross microscopy techniques, which permit the direct visualization of the biofilms, while indirect methods estimate the numbers of adherent microbes in situ (6, 9) or require the detachment of the microbes from the substratum and their quantification (25). Indirect methods with 96-well microtiter plates, which permit the simultaneous quantification of the yeasts in large numbers of biofilms, have been recommended by many workers due to their simplicity and the sensitivity. A reduction assay with the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) is one such technique that has been proven to be very popular (8, 17, 33). It is a colorimetric method that uses XTT, which is reduced to the XTT formazan product by the mitochondrial dehydrogenases of metabolically active yeast cells. The colorimetric change so produced is proportional to the number of living cells and can be quantified (8, 17, 33). In the crystal violet assay, the biofilms are stained with crystal violet, followed by decolorization (15). The absorbance of the decolorization solution, which reflects the numbers of viable cells, can then be measured colorimetrically with a microtiter plate reader (6).

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Although previous workers have used these techniques for biofilm assays, their reliabilities have not been compared. Hence, we also compared the biofilm formation of 46 *C. albicans* isolates using these two methods. However, the primary aim of the study was to compare quantitatively the biofilm-forming abilities of *C. albicans* isolates from HIV-infected and HIV-free subjects. Furthermore, host factors such as CD4-cell count and intake of zidovudine were evaluated for their effects, if any, on candidal biofilm production.

MATERIALS AND METHODS

Organisms. The *C. albicans* isolates used in this study included 20 oral isolates from HIV-free individuals and 26 oral isolates from HIV-infected patients. The isolation and identification of *Candida* from HIV-infected and healthy individuals were performed as described previously (28). All 46 isolates were subcultured from thawed suspensions of pure isolates stored at the Oral Bioscience Laboratory at the Prince Philip Dental Hospital, University of Hong Kong. The identities of all isolates were reconfirmed by the standard germ tube test and the commercially available API 20C-AUX method (Bio-Merieux, Marcy l'Etoile, France).

Growth conditions. All *C. albicans* isolates were cultured at 37°C for 18 h in Sabouraud's dextrose agar. A loopful of the yeasts was then inoculated in yeast nitrogen base medium supplemented with 100 mM glucose. After overnight culture of the broth, the yeasts were harvested in the late exponential growth phase, washed twice with 5 ml of phosphate-buffered saline (PBS; pH 7.2; Ca^{2+}- and Mg^{2+}-free), and suspended to 10^7 cells/ml by adjusting the optical density of the suspension to 0.38 at 520 nm. This cell concentration was selected because previous workers have demonstrated that optimal biofilm formation occurs at this particular density (16). The standardized cell suspension was used immediately.

Biofilm formation. Biofilms were produced on commercially available presterilized, polystyrene, flat-bottom 96-well microtiter plates (Iwaki, Tokyo, Japan). At first, 100 μl of a standardized cell suspension (10^7cells/ml) was transferred into each well of a microtiter plate with a pipette, and the plate was incubated for 1.5 h at 37°C in a shaker at 75 rpm to allow the yeast to adhere to the surfaces of the wells (16). As controls, three wells of each microtiter plate were handled in an identical fashion, except that no *Candida* suspensions were added.

Following the adhesion phase, the cell suspensions were aspirated and each well was washed twice with 150 μl of PBS to remove loosely adherent cells. A total of 100 μl of yeast nitrogen base medium was then transferred into each of the washed wells with a pipette, and the plates were incubated at 37°C in a shaker at 75 rpm. The biofilms were allowed to develop for up to 66 h, and then the yeasts were quantified by both the XTT reduction assay and the crystal violet assay (see below). The medium was replenished daily by aspiration of the spent medium and the addition of fresh medium. All assays were carried out on three different occasions in triplicate.

XTT reduction assay. The XTT reduction assay used was a modification of the methods described previously (17, 25). Briefly, XTT (Sigma, St. Louis, Mo.) solution (1 mg/ml in PBS) was prepared, filter sterilized through a 0.22-μm-pore-size filter, and stored at −70°C. Menadione (Sigma) solution (0.4 mM) was prepared and filter sterilized immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume.

The biofilms were first washed five times with 200 μl of PBS, and then 200 μl of PBS and 12 μl of the XTT-menadione solution were added to each of the prewashed wells and the control wells. The microtiter plate was then incubated in the dark for 2 h at 37°C. Following incubation, 100 μl of solution was transferred to new wells and the color change in the solution was measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd., Sunnyvale, Calif.) at 490 nm. The absorbance values for all new wells were preread at 490 nm, since their levels of absorbance may display deviations. The absorbance values for the controls were then subtracted from the values for the test wells to eliminate spurious results due to background interference.

A total of 18 *Candida* isolates (9 isolates each from the HIV-infected and the HIV-free groups) were randomly selected from the pool of 46 isolates to evaluate the XTT activities of the cells in the planktonic phase. For this purpose, 12 μl of the XTT-menadione solution was added to 200 μl of the freshly grown (see above) cell suspensions, and the cell suspensions were incubated in the dark for 2 h. The cell suspensions were centrifuged (6,000 × *g* for 10 min), and the optical density of the supernatants were measured as described above.

Crystal violet staining. Biofilm formation was also quantified by a modification of a crystal violet assay recently described by others (6). Briefly, the biofilm-coated wells of microtiter plates were washed twice with 200 μl of PBS and then air dried for 45 min. Then, each of the washed wells was stained with 110 μl of 0.4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed four times with 350 μl of sterile distilled water and immediately destained with 200 μl of 95% ethanol. After 45 min of destaining, 100 μl of destaining solution was transferred to a new well and the amount of the crystal violet stain in the destaining solution was measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd.) at 595 nm. The absorbance values for the controls were subtracted from the values for the test wells to minimize background interference.

Statistical analysis. The data on the biofilm formation abilities of the different isolates were normalized with the data for isolate 191354g, which was found to be a good biofilm former in a previous pilot study. To derive associations between host factors and *Candida* biofilm formation, a general linear model of SPSS software (version 10.0) was adopted. A backward elimination was performed so that only relevant factors were left for the final analyses. Assessment of the linearity of the results of the XTT reduction and crystal violet assays (for HIV-infected and -free groups) was also performed with SPSS software. *P* values of <0.05 were considered statistically significant.

RESULTS

XTT reduction by planktonic yeast cultures of various isolates. A preliminary study was conducted with 18 randomly selected *C. albicans* isolates (from the pool of 46 isolates) in order to test the homogeneity of the XTT activity of each of the isolates, which is a prerequisite for valid interstrain comparison of biofilm-forming ability. The 18 isolates comprised 9 isolates each from HIV-infected and -free individuals. The XTT activities were normalized to the XTT activity of isolate 191354g (16). As seen in Fig. 1, all *C. albicans* isolates displayed consistent and similar values, and one-way analysis of variance showed no significant difference between the XTT activities of the 18 isolates ($P = 0.202$). An independent-samples *t* tests further confirmed the absence of a significant difference in biofilm formation between the isolates derived from HIV-infected and -free individuals ($P = 0.328$).

In another series of experiments, an attempt was made to correlate the XTT activity with the number of planktonic yeasts. Statistical analyses showed that XTT activity was linearly associated with the log of the cell concentration over the cell concentration range tested (from 2×10^5 to 10^8 cells/ml) ($P = 0.001$; correlation coefficient = 0.977) (Fig. 2). Thus, it follows that XTT activity can be used as an indicator of cell numbers in a cell suspension.

Comparison of XTT reduction and crystal violet assays. When the XTT reduction and the crystal violet assays were performed with the 46 isolates on three different occasions in triplicate, the coefficient of variation (CV) of the crystal violet assay was significantly greater than that of the XTT reduction assay (CV of the XTT reduction assay = 0.321; CV of the crystal violet assay = 0.442 [$P = 0.025$ by the paired-samples *t* test]). An effort was also made to correlate the results of the XTT reduction and crystal violet assays. The correlation analyses showed a significant positive relationship between the two methods, despite the isolate source (for isolates from HIV-free subjects, correlation coefficient = 0.833 and $P < 0.001$; for isolates from HIV-positive patients, correlation coefficient = 0.757 and $P < 0.001$) (Fig. 3) although as noted above, the

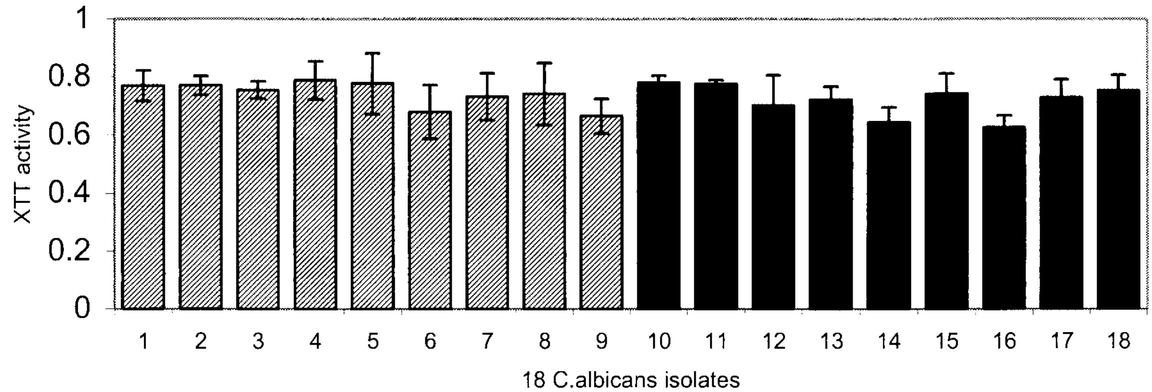

FIG. 1. XTT activities of 18 clinical *C. albicans* isolates. Isolates 1 to 9 were from HIV-free individuals, and isolates 10 to 18 were from HIV-infected individuals. XTT activities were measured colorimetrically at 490 nm with a microtiter plate reader. The results were normalized to the activity of isolate 191354g. Results represent means ± standard deviations (error bars) from three independent experiments.

XTT reduction assay was more reliable than the crystal violet assay.

Comparison of biofilm formation abilities of *C. albicans* isolates from HIV-infected and HIV-free patients. Using the XTT reduction and crystal violet assays, we compared 26 isolates from HIV-infected patients and 20 isolates from HIV-free patients for their abilities to produce biofilms in vitro. By statistical analysis (independent-sample t test), neither the XTT reduction assay ($P = 0.340$) nor the crystal violet assay ($P = 0.585$) revealed significant differences in biofilm formation abilities between the two groups of isolates. As measured by the XTT reduction assay, the levels of biofilm formation by the 46 *C. albicans* isolates from HIV-infected and HIV-free individuals ranged from 0.09941 to 1.0588 (mean ± standard error of the mean [SEM], 0.4841 ± 0.0588) and 0.1527 to 1.0000 (mean ± SEM, 0.4546 ± 0.0609), respectively. The corresponding levels determined by the crystal violet assay ranged from 0.0978 to 1.0208 (mean ± SEM, 0.1956 ± 0.0720) and

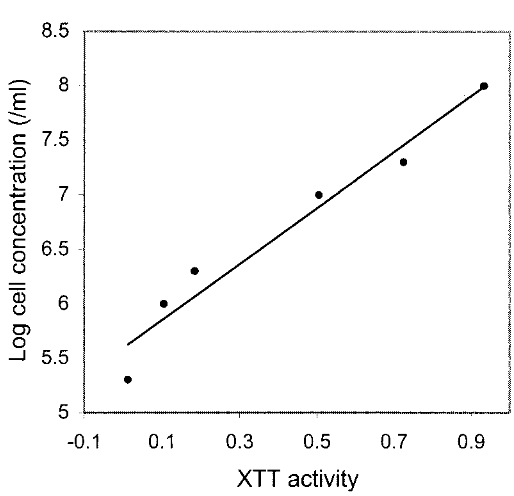

FIG. 2. Relationship between XTT reduction assay and log cell concentration evaluated from CFU counts (for isolate 191354g) (correlation coefficient = 0.977; $P = 0.001$).

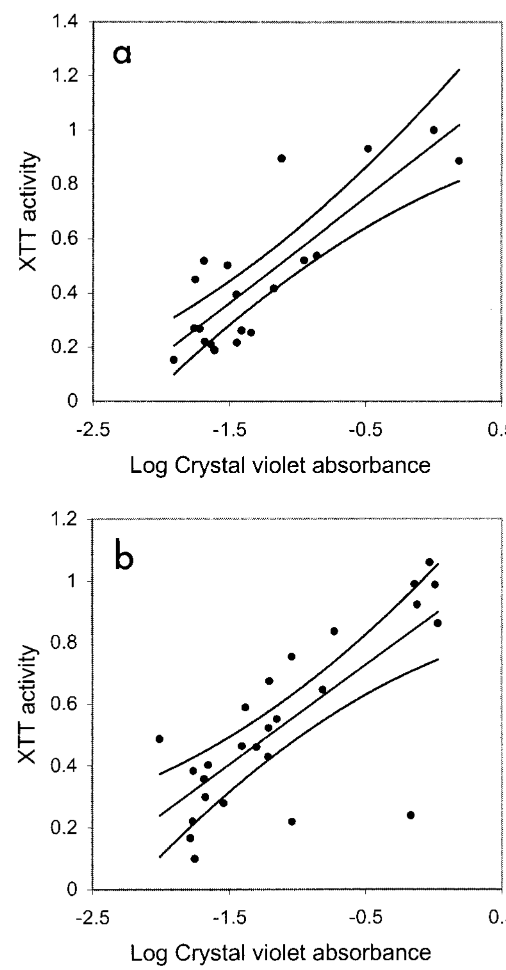

FIG. 3. Scatter plot showing the relationship between the results of the crystal violet and XTT reduction assays. The data were derived from biofilms generated by 20 *C. albicans* isolates from HIV-free subjects (a) and 26 *C. albicans* isolates from HIV-infected patients (b). Curves represent the 95% confidence intervals of the regression lines. Each value is the mean of three independent experiments. All results were normalized to the value for isolate 191354g.

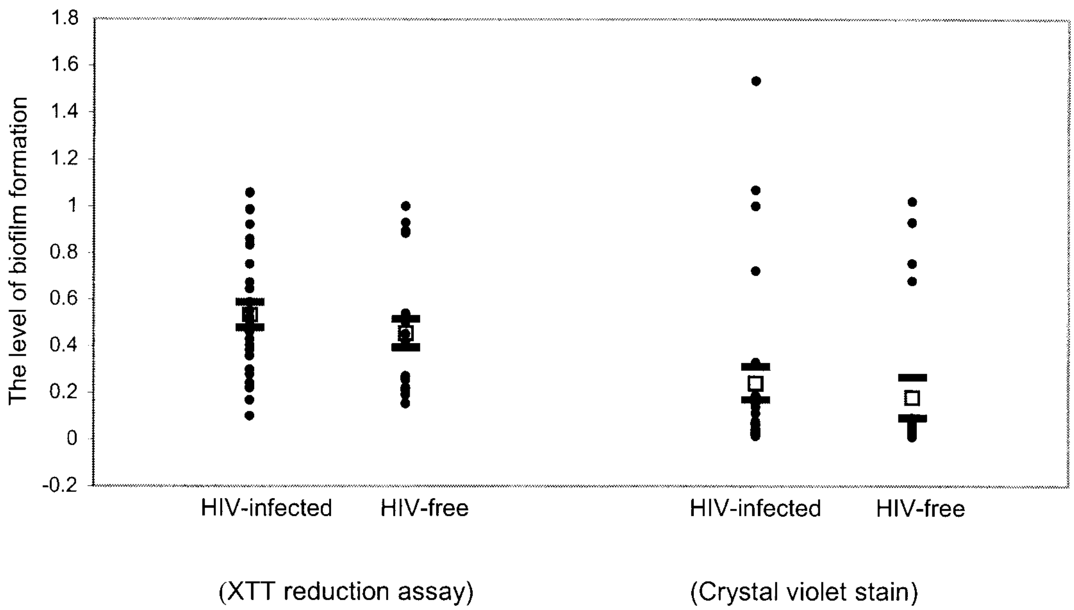

FIG. 4. Biofilm formation by *C. albicans* isolates from HIV-infected and HIV-free individuals evaluated by two independent colorimetric methods. Bars represent the SEMs. For HIV-infected versus HIV-free individuals, P was equal to 0.729 as determined by the XTT reduction assay and P was equal to 0.889 as determined by the crystal violet assay.

0.01237 to 1.5350 (mean $\pm$ SEM 0.1757 $\pm$ 0.0869) for the HIV-infected and HIV-free groups, respectively (Fig. 4).

Association of host factors with *Candida* biofilm formation. By using a general linear model of SPSS software (version 10.0), the possible associations of several host factors with biofilm formation by *C. albicans* isolates from HIV-infected patients were investigated. Initially, the factors tested included the presence of oral candidiasis; age; CD4-cell count; Centers for Disease Control and Prevention (CDC) staging; and intake of zidovudine and antifungal, antibacterial, and antiparasitic medications. A backward elimination process was then conducted to exclude irrelevant host factors. After this procedure was performed, three host factors including age, CD4-cell count, and intake of zidovudine were left for the final association analyses. The results of the analyses are summarized in Table 1.

The isolates from the patients older than age 35 years had a significantly lower degree of biofilm formation than those from the patients younger than age 35 years, according to the results of the crystal violet assay ($P = 0.015$), although the difference in the degree of biofilm formation was not deemed significant according to the results of the XTT reduction assay ($P = 0.201$). A lower degree of biofilm formation was also associated with isolates from patients who had CD4 counts greater than 350 cells/μl at the time of yeast collection (for the XTT reduction assay, $P = 0.02$; for the crystal violet assay, $P = 0.007$). The *Candida* isolates from the patients who were receiving zidovudine at the time of sampling were found to have a significantly higher degree of biofilm formation than isolates from those who were not receiving this drug (for the XTT reduction assay, $P = 0.001$; for the crystal violet assay, $P = 0.000$).

Factors including the presence of candidasis, CDC staging, and intake of antifungal, antibacterial, and antiparasitic drugs were not found to be significantly associated with *Candida* biofilm formation ($P > 0.05$ for all comparisons).

DISCUSSION

A variety of model systems have been used to evaluate *Candida* biofilm formation. The most commonly used methods include the microtiter plate systems with colorimetric assessment (6, 16), chemostat-based systems (10, 31), and perfused biofilm fermentors (11). Of these, the microtiter plate-based systems are simple and inexpensive yet permit evaluation of

TABLE 1. Associations of three host factors with biofilm formation by 26 *C. albicans* isolates from HIV-infected patients[a]

Host factor	No. of patients	Estimated marginal mean		P value	
		XTT reduction assay	Crystal violet assay	XTT reduction assay	Crystal violet assay
Age (yr)					
<35	14	0.626	0.412	0.201	0.015[b]
>35	12	0.519	0.190		
$CD4^+$ count (no. of cells/μl)					
<350	16	0.676	0.427	0.02[b]	0.007[b]
>350	10	0.468	0.175		
Zidovudine intake					
No	18	0.399	0.046	0.001[b]	0.000[b]
Yes	8	0.745	0.556		

[a] A general linear model of SPSS software (version 10.0) was used.
[b] A significant difference at a P value of <0.05.

the effects of multiple factors on biofilm formation and therefore are practical for the screening of a large numbers of isolates. Hence, in the present study we used a modified microtiter plate-based model system to assess the biofilm formation abilities of 46 *C. albicans* isolates. Although the XTT reduction and crystal violet assays have been extensively used in the past to evaluate microbial biofilms in microtiter plate systems, ours appears to be the first report that comprehensively compares these two quantitative techniques.

XTT is a water-soluble yellow salt and can be reduced by mitochondrial dehydrogenases of metabolically active yeast cells. The color change during the reaction reflects the number of viable cells in the sample and can be quantified by a microtiter plate reader or a spectrophotometer (8, 17, 33). A linear relationship between incubation time and color development has been noted when the incubation duration is no longer than 5 h (2, 3). Samples are usually incubated with XTT for only about 2 h to permit a color change and to save experimental time.

On the other hand, there are some pitfalls with the XTT reduction assay. It is well known that cells in biofilms display properties dramatically different from those of their free-living counterparts (4). These surface-associated changes may include alterations in XTT metabolism by biofilm cells. Additionally, the rate of metabolism of XTT may vary from species to species, as it has been shown that the rate of metabolism of XTT by *Candida parapsilosis* is slower than that by *C. albicans* (16). Interestingly, even within the same species, noninvasive *Candida* strains have been found to show higher levels of XTT metabolic activity than invasive strains (16). XTT metabolism can also be influenced by external factors, as there is evidence that pretreatment of yeasts with antifungals can increase the level of XTT metabolism and, consequently, can lead to overestimation of the degree of biofilm formation (17). Thus, caution must be exercised when the XTT reduction assay is used to quantify yeast biofilm production.

It is also unclear whether different strains of a given *Candida* species have equal XTT activities. To ascertain this, we prepared standardized cell suspensions (10^7 cells/ml) of 18 different *C. albicans* isolates and found no significant difference in the XTT activities of the strains tested ($P = 0.202$). Our data also showed no difference in XTT activities between isolates from HIV-infected and HIV-free individuals ($P = 0.328$). Further studies revealed a linear relationship between XTT activity and the log of the yeast cell concentration within the range of cell concentrations tested (correlation coefficient = 0.977; $P = 0.001$). This positive correlation persisted with isolates from both HIV-infected (coefficient of correlation = 0.757; $P < 0.001$) and HIV-free individuals (correlation coefficient = 0.833; $P < 0$). Finally, our data indicated a significant correlation between the XTT reduction assay and the crystal violet assay for the quantification of biofilm formation, although the former demonstrated a higher degree of reliability.

It is well known that, in comparison with healthy controls, HIV-infected individuals have an increased incidence of symptom-free oral *C. albicans* carriage and a heightened frequency of oral candidiasis (27). One possible reason for this may be the heightened biofilm-forming abilities of the *Candida* isolates colonizing the oral cavities of HIV-infected individuals. However, our data on candidal biofilm formation, obtained by comparison of a large group of isolates from healthy and HIV-infected individuals and by two independent assay techniques, could not demonstrate significant differences in biofilm-forming abilities between the isolates recovered from HIV-infected individuals and those recovered from HIV-free individuals. Because in our study in vitro biofilm formation was done on an inert surface, which excludes environmental effects, only the characteristics of the isolates examined account for our results. It could therefore be argued that HIV infection per se does not necessarily alter the biofilm-forming characteristics of commensal oral *C. albicans* isolates. This is in agreement with the findings of other workers, who reported comparable levels of adherence to BECs by isolates from HIV-infected and healthy individuals (34).

Two other hypotheses may explain the increased rates of *C. albicans* carriage and the heightened frequency of oral candidiasis in HIV-infected individuals compared with those in healthy controls. One possible reason for this phenomenon is undoubtedly the compromised immune systems of HIV-infected individuals, as well as possible alterations in the quality of host mucosal cells and the oral environment. A second possible reason is that the *Candida* population itself may modify its virulence attributes other than biofilm-forming ability as a consequence of HIV infection. The first hypothesis is supported to some extent by the presence of HIV type 1 provirus DNA in the nuclei of saliva-associated epithelial cells in HIV-infected patients (24), which in turn may alter their receptivities, as some have shown that *C. albicans* cells better adhere to BECs from HIV-positive patients than to cells from healthy individuals (34). As for the second hypothesis, there is evidence that, in comparison to commensal isolates from healthy individuals, *Candida* strains isolated from HIV-infected patients show increased levels of expression of secreted aspartyl proteinases (22, 37) and increased levels of resistance to antifungals (28), adhere to BECs better (32), and exhibit elevated rates of phenotypic switching (36).

We also examined the associations between a number of common host variables and candidal biofilm formation. When the intake of zidovudine, a common antiretroviral drug, was investigated for its association with biofilm-forming ability, a higher degree of biofilm formation was seen among isolates from the group that was receiving zidovudine (for the XTT reduction assay, $P = 0.001$; for the crystal violet assay, $P = 0.000$). Interestingly, previous workers (20) have reported a higher frequency of candidiasis in association with the use of zidovudine. Similar results have also been obtained in an in vitro study by other workers (34), who observed a positive association between the use of zidovudine and the degree of candidal adherence to BECs ($P < 0.0001$). This increased adhesion was attributed to zidovudine-related xerostomia, anemia, and neutropenia (7, 18). Thus, it is tempting to speculate that the intake of zidovudine may be associated with a heightened ability of yeasts to produce biofilms, as shown in the present study, and an increase in the receptivities of host cells for yeasts, as shown previously (34). Such enhanced biofilm-forming ability may also be linked to strain replacement (35) and/or genotypic changes (31) in *Candida* species as a consequence of zidovudine intake.

The data from the present study also revealed a higher degree of biofilm formation by isolates from patients who had

CD4 counts lower than 350 cells/μl (for the XTT reduction assay, $P = 0.02$; for the crystal violet assay, $P = 0.007$). This implies that immune suppression leads to the selection of a resident oral yeast flora that possesses a greater proclivity to biofilm formation. This in vitro observation certainly concurs well with the increased prevalence of pseudomembranous candidiasis in association with immune incompetence in the late stages of HIV infection and AIDS. Indeed, these pseudomembranes could be considered archetypal, well-developed, complex yeast biofilms, although this concept has not been well recognized.

No significant differences in biofilm formation were found when isolates from the oral candidiasis-free group were compared with those from the oral candidiasis group ($P > 0.05$), although previous workers have observed the replacement of candidal strains and species and "genotypic shuffling" during the development of oral candidasis in HIV-infected patients (28a, 35).

Our data also showed that the isolates from patients who were on antifungals produced biofilms not significantly different from those produced by the control isolates ($P > 0.05$). The available data on this issue are inconsistent. Some have reported that the treatment of the yeast cells with antifungals reduced candidal adhesion in vitro (19), while in another study, neither candidal adhesion nor the receptivity of yeasts to BECs was affected by the use of antifungals (34). Hence, further research is needed to confirm or refute our findings.

Of the other variables examined, the CDC stage of the patients and the use of antibacterial and antiparasitic drugs were not associated with biofilm formation ($P > 0.05$ for all comparison). This concurs with the findings of other workers (34), who also found no relationship between these variables and the adherence of *C. albicans* to BECs in vitro.

In conclusion, the cause of the increased prevalence of candidal carriage and candidiasis in HIV-infected individuals is multifactorial and is unlikely to be related to the biofilm-forming abilities of the yeast isolates that either reside in the oral cavity or cause disease. However, our data point to some host attributes that may be associated with candidal biofilm formation. In vivo studies, possibly with animal models, are warranted to substantiate these findings.

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REFERENCES

1. **Branchini, M. L., M. A. Pfaller, J. Rhine-Chalberg, T. Frempong, and H. D. Isenberg.** 1994. Genotypic variation and slime production among blood and catheter isolates of *Candida parapsilosis*. J. Clin. Microbiol. **32:**452–456.
2. **Buttke, T. M., J. A. McCubrey, and T. C. Owen.** 1993. Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. J. Immunol. Methods **157:**233–240.
3. **Cory, A. H., T. C. Owen, J. A. Barltrop, and J. G. Cory.** 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. **3:**207–212.
4. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
5. **Cox, F.** 1983. Adherence of *Candida albicans* to buccal epithelial cells in children and adults. J. Lab. Clin. Med. **102:**960–972.
6. **Djordjevic, D., M. Wiedmann, and L. A. McLandsborough.** 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl. Environ. Microbiol. **68:**2950–2958.
7. **Ferguson, M. M.** 1975. Oral mucous membrane markers of internal disease: part II, p. 246–250. *In* A. E. Dolby (ed.), Oral mucosa in health and disease. Blackwell Scientific Publications, Oxford, United Kingdom.
8. **Hawser, S.** 1996. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronized *C. albicans* cells. J. Med. Vet. Mycol. **34:**149–152.
9. **Hawser, S. P., and L. J. Douglas.** 1994. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect. Immun. **62:**915–921.
10. **Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov, and M. R. Parsek.** 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. J. Bacteriol. **83:**5395–5401.
11. **Hodgson, A. E., S. M. Nelson, M. R. Brown, and P. Gilbert.** 1995. A simple *in vitro* model for growth control of bacterial biofilms. J. Appl. Bacteriol. **79:**87–93.
12. **Kennedy, M. J.** 1988. Adhesion and association mechanisms of *Candida albicans*. Curr. Top. Med. Mycol. **2:**731–769.
13. **Kennedy, M. J., and P. A. Volz.** 1985. Ecology of *Candida albicans* gut colonization: inhibition of *Candida* adhesion, colonization, and dissemination from the gastrointestinal tract by bacterial antagonism. Infect. Immun. **49:**654–663.
14. **Kontoyiannis, D. P., and R. E. Lewis.** 2002. Antifungal drug resistance of pathogenic fungi. Lancet **359:**1135–1144.
15. **Kruczak-Filipov, P., and P. Shively.** 1992. Gram stain procedure, p. 1.5.1–1.5.18. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook, vol. 1. American Society for Microbiology, Washington, D.C.
16. **Kuhn, D. M., J. Chandra, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. Infect. Immun. **70:**878–888.
17. **Kuhn, D. M., T. George, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob. Agents Chemother. **46:**1773–1780.
18. **MacFarlane, T. W., and D. K. Mason.** 1975. The physiological responsiveness of the oral mucosa: the role of saliva, p. 127–130. *In* A. E. Dolby (ed.), Oral mucosa in health and disease. Blackwell Scientific Publications, Oxford, United Kingdom.
19. **Macura, A. B.** 1988. The influence of some antifungal drugs on in vitro adherence of *Candida albicans* to human buccal epithelial cells. Mycoses **31:**71–76.
20. **McCarthy, G. M., I. D. Mackie, J. Koval, H. S. Sandhu, and T. D. Daley.** 1991. Factors associated with increased frequency of HIV-related oral candidasis. J. Oral Pathol. Med. **20:**332–336.
21. **Nguyen, M. H., J. E. Peacock, D. C. Tanner, A. J. Morris, M. L. Nguyen, D. R. Snydman, M. M. Wagener, and V. L. Yu.** 1995. Therapeutic approaches in patients with candidemia. Evaluation in a multicenter, prospective, observational study. Arch. Intern. Med. **155:**2429–2435.
22. **Ollert, M. W., C. Wende, M. Gorlich, C. G. McMullan-Vogel, M. Borg-von Zepelin, C. W. Vogel, and H. C. Korting.** 1995. Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. J. Clin. Microbiol. **33:**2543–2549.
23. **Pereiro, M., Jr., A. Losada, and J. Toribio.** 1997. Adherence of *Candida albicans* strains isolated from AIDS patients. Comparison with pathogenic yeasts isolated from patients without HIV infection. Br. J. Dermatol. **137:**76–80.
24. **Qureshi, M. N., C. E. Barr, T. Seshamma, J. Reidy, R. J. Pomerantz, and O. Bagasra.** 1995. Infection of oral mucosal cells by human immunodeficiency virus type 1 in seropositive persons. J. Infect. Dis. **171:**190–193.
25. **Ramage, G., K. V. Walle, B. L. Wickes, and J. L. López-Ribot.** 2001. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob. Agents Chemother. **45:**2475–2479.
26. **Samaranayake, L. P., L. K. Cheung, and Y. H. Samaranayake.** 2002. Candidiasis and other fungal diseases of the mouth. Dermatol. Ther. **15:**252–270.
27. **Samaranayake, L. P., P. L. Fidel, J. R. Naglik, S. P. Sweet, R. Teanpaisan, and M. M. Coogan.** 2002. Fungal infections associated with HIV infection. Oral Dis. **8:**151–160.
28. **Samaranayake, Y. H., L. P. Samaranayake, E. Pow, and N. Beena.** 2001. The antifungal effect of lysozyme and lactoferrin against genetically similar, sequential *Candida albicans* isolates from a human immunodeficiency virus-infected southern Chinese cohort. J. Clin. Microbiol. **39:**3296–3302.

28a. **Samaranayake, Y. H., L. P. Samaranayake, J. Y. Y. Yau, W. K. Tsang, B. P. K. Cheung, and K. W. S. Yeung.** 2003. "Genotypic shuffling" of sequential clones of *C. albicans* in HIV-infected individuals with and without symptomatic oral candidiasis. J. Med. Microbiol. **52:**349–359.

29. **Sanglard, D., and F. C. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. **2:**73–85.
30. **Singh, P. K., M. R. Parsek, E. P. Greenberg, and M. J. Welsh.** 2002. A

component of innate immunity prevents bacterial biofilm development. Nature **417:**552–555.
31. **Singh, S., J. D. Sobel, P. Bhargava, D. Boikov, and J. A. Vazquez.** 2002. Vaginitis due to *Candida krusei*: epidemiology, clinical aspects, and therapy. Clin. Infect. Dis. **35:**1066–1070.
32. **Sweet, S. P., S. Cookson, and S. J. Challacombe.** 1995. *Candida albicans* isolates from HIV-infected and AIDS patients exhibit enhanced adherence to epithelial cells. J. Med. Microbiol. **43:**452–457.
33. **Tellier, R., M. Krajden, G. A. Grigoriew, and I. Campbell.** 1992. Innovative endpoint determination system for antifungal susceptibility testing of yeasts. Antimicrob. Agents Chemother. **36:**1619–1625.
34. **Tsang, C. S., and L. P. Samaranayake.** 1999. Factors affecting the adherence of *Candida albicans* to human buccal epithelial cells in human immunodeficiency virus infection. Br. J. Dermatol. **141:**852–858.
35. **Vargas, K., and S. Joly.** 2002. Carriage frequency, intensity of carriage, and strains of oral yeast species vary in the progression to oral candidiasis in human immunodeficiency virus-positive individuals. J. Clin. Microbiol. **40:**341–350.
36. **Vargas, K., S. A. Messer, M. Pfaller, S. R. Lockhart, J. T. Stapleton, J. Hellstein, and D. R. Soll.** 2000. Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. J. Clin. Microbiol. **38:**3595–3607.
37. **Wu, T., L. P. Samaranayake, B. Y. Cao, and J. Wang.** 1996. *In vitro* proteinase production by *Candida albicans* isolates from individuals with and without HIV infection and its attenuation by antimycotics. J. Med. Microbiol. **44:**311–316.